MOLECULAR AND CELLULAR BIOLOGY, Mar. 1995, p. 1162–1168 Vol. 15, No. 3
0270-7306/95/$04.00+0

Thyrotropin-Induced Mitogenesis Is Ras Dependent but Appears To Bypass the Raf-Dependent Cytoplasmic Kinase Cascade

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Received 26 August 1994/Returned for modification 29 September 1994/Accepted 25 November 1994

Cellular growth control requires the coordination and integration of multiple signaling pathways which are likely to be activated concomitantly. Mitogenic signaling initiated by thyrotropin (TSH) in thyroid cells seems to require two distinct signaling pathways, a cyclic AMP (cAMP)-dependent signaling pathway and a Ras-dependent pathway. This is a paradox, since activated cAMP-dependent protein kinase disrupts Ras-dependent signaling induced by growth factors such as epidermal growth factor and platelet-derived growth factor. This inhibition may occur by preventing Raf-1 protein kinase from binding to Ras, an event thought to be necessary for the activation of Raf-1 and the subsequent activation of the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinases (MEKs) and MAP kinase (MAPK)/ERKs. Here we report that serum-stimulated hyperphosphorylation of Raf-1 was inhibited by TSH treatment of Wistar rat thyroid cells, indicating that in this cell line, as in other cell types, increases in intracellular cAMP levels inhibit activation of downstream kinases targeted by Ras. Ras-stimulated expression of genes containing AP-1 promoter elements was similarly inhibited by TSH. On the other hand, stimulation of thyroid cells with TSH resulted in stimulation of DNA synthesis which was Ras dependent but both Raf-1 and MEK independent. We also show that Ras-stimulated DNA synthesis required the use of this kinase cascade in untreated quiescent cells but not in TSH-treated cells. These data suggest that in TSH-treated thyroid cells, Ras might be able to signal through effectors other than the well-studied cytoplasmic kinase cascade.

Cross talk between different signaling systems plays an important role in signal cascades initiated by a variety of growth factors. Thyrotropin, or thyroid-stimulating hormone (TSH), is one such growth factor. The receptor for TSH is a seven-membrane-spanning receptor which couples to the heterotrimeric G protein G_S (58). Injection of an affinity-purified antibody to G_S abolished TSH-induced DNA synthesis in Wistar rat thyroid (WRT) cells (41). Binding of TSH to its receptor results in activation of G_S and adenylate cyclase and generation of the intracellular second messenger cyclic AMP (cAMP), activating the cAMP-dependent protein kinase (PKA). PKA appears to be required for TSH-induced mitogenesis, since microinjection of the heat-stable protein kinase inhibitor (PKI) of PKA significantly reduces TSH-induced DNA synthesis in WRT cells (31). Many of the effects of TSH on thyroid cells can be mimicked by cAMP agonists, further substantiating the role of PKA in TSH signaling (15). In addition, microinjection of a dominant-interfering Ras protein (N17 Ras) reduces TSH-stimulated DNA synthesis in WRT cells, as does injection of the Ras GTPase-activating protein GAP and the Ras effector-specific antibody Y13-259, indicating that activation of Ras is required for TSH-induced mitogenesis (2, 30, 31). Microinjection of both PKI and dominant-negative Ras together into WRT cells reduces TSH-stimulated DNA synthesis to a level lower than that with injection of either inhibitor alone, further suggesting that the full mitogenic response to TSH requires both signaling pathways (31). Further, activating mutations in both G_S and Ras are frequently found in thyroid tumors (53, 54).

It is an apparent paradox that both Ras and PKA cooperate in TSH signaling, since one of the examples of negative cross talk between signaling pathways is that between Ras- and cAMP-dependent signaling pathways. In several cell types in which increases in intracellular cAMP levels inhibit growth, cAMP exerts a negative effect on Ras-mediated signaling pathways. Ras seems to play a role in the activation of Raf-1 through localization of Raf-1 to the plasma membrane (34, 52). Much biochemical and genetic data indicate that activation of Raf and the subsequent activation of the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK) and MAP kinases (the Raf-dependent cytoplasmic kinase cascade) are required or at least involved in Ras-dependent signaling pathways in a variety of organisms (1, 45). This cytoplasmic kinase cascade links Ras activation at the plasma membrane to changes in gene expression in the nucleus (25). Recent work has demonstrated that one way in which elevations in cAMP levels probably repress growth is by inhibiting the coupling of Ras to its downstream effector, Raf-1 (7, 10, 20, 50, 60).

In primary dog thyrocytes, TSH stimulates proliferation, and yet treatment does not induce the phosphorylation or nuclear translocation of MAP kinase (33). In several cell types, MAP kinase translocates to the nucleus upon its activation and phosphorylates and activates a variety of transcription factors such as TCF/Elk-1, thus playing a role in the induction of immediate-early genes (18, 19, 35, 38, 49). Overexpression of wild-type

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H-Ras by microinjection in WRT cells leads to DNA synthesis and the expression of AP-1-controlled genes, but treatment of cells with TSH does not stimulate the expression of genes regulated by AP-1 promoter elements (56). In fact, treatment of cells with either TSH or cAMP agonists represses both insulin-like growth factor 1 and fetal calf serum (FCS)-stimulated expression of c-*jun*, c-*fos*, and *egr-1* mRNA, as well as serum- and phorbol ester-stimulated expression of AP-1-controlled genes (56). These data suggest that signaling initiated by TSH, although Ras dependent, may not occur through the well-studied cytoplasmic kinase cascade which leads to expression of AP-1-controlled genes.

In this study, we have further assessed the role of the Raf-dependent cytoplasmic kinase cascade in TSH-induced mitogenic signaling. Treatment of cells with TSH and cAMP agonists inhibited activation of the Raf-dependent cytoplasmic kinase cascade by a variety of agents including Ras. We microinjected Raf-1 antibodies and dominant-interfering MEK proteins into WRT cells and found that TSH-induced DNA synthesis, which is known to be Ras dependent, appeared to be Raf-1 and MEK independent. In contrast, mitogenesis resulting from injection of quiescent cells with cellular H-Ras protein was Raf-1 and MEK dependent. Moreover, stimulation of WRT cells with cAMP-elevating agents apparently channeled Ras-dependent signaling from the well-studied kinase cascade to an undefined alternate pathway, suggesting that Ras might be able to mediate TSH signaling through an effector(s) other than Raf-1 and MEK in thyroid cells.

MATERIALS AND METHODS

Cell culture and microinjection. Rat1 cells expressing insulin receptors as a result of stable transfection (39) were cultured on glass coverslips in Dulbecco's modified Eagle/F12 medium (Cell Grow) supplemented with 10% FCS. At between 24 and 36 h prior to microinjection, the cells were rendered quiescent by starvation in serum-free medium. WRT cells were cultured in a six-hormone-containing medium (6H [3]). At 48 h prior to microinjection, the cells were rendered quiescent by starvation in Coon's modified Ham's F12 medium containing 0.3% bovine serum albumin and 0.5 μg of insulin per ml. For gene expression studies, we used WRT cells stably transfected with the reporter gene *lacZ* under the regulation of two copies of an AP-1 promoter element (WRT-TRE cells) (56). WRT-TRE cells were grown in 6H further supplemented with 150 μg of Geneticin per ml. Individual cells were microinjected with an Eppendorf automated micromanipulator (model 5171). In a typical experiment, 200 to 300 cells were injected per coverslip. Purified proteins were injected at the following concentrations: affinity-purified Raf-1 antibodies (URP30K) (29), 5 mg/ml; rabbit or mouse immunoglobulin G (IgG), 5 mg/ml; wild-type (wt) H-Ras (following expression and purification from *Escherichia coli* [21]), 95 μM; and K97M MEK proteins, 28 μM (37).

Analysis of gene expression and DNA synthesis. For gene expression, WRT-TRE cells were injected with proteins as described above and fixed in 3.7% formaldehyde 12 to 18 h after protein injection. The cells were stained for *lacZ* gene expression by incubation for 2 to 4 h in a phosphate-buffered saline (PBS) solution containing 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml, 5 mM ferrocyanide, 5 mM ferricyanide, and 2 mM $MgCl_2$. Injected cells were detected with a fluorescein isothiocyanate-coupled anti-rabbit or -mouse antibody (Jackson Laboratories). For DNA synthesis, 2 to 3 h after injection, the nucleotide analog bromodeoxyuridine (BrdU; Amersham) was added to the media, together with 20% FCS, 100 ng of insulin per ml, 1 μg of epidermal growth factor (EGF) per ml, 1 mM 8-bromo-cAMP (8Br-cAMP), or 10 mU of TSH per ml unless stated otherwise. At 18 h after injection, the Rat1 cells were fixed with 3.7% formaldehyde. BrdU incorporation into newly synthesized DNA was detected by staining with a rat monoclonal antibody (Sera Labs), followed by staining with a rhodamine-conjugated anti-rat antibody. At 48 h after injection of the WRT cells, cells were fixed with ethanol-acetic acid (95:5) for 30 min. BrdU incorporation into newly synthesized DNA was detected by staining with a mouse monoclonal BrdU antibody (Amersham) and then with a rhodamine-coupled anti-mouse antibody (Jackson Laboratories). Injected cells were detected with fluorescein-conjugated antibodies as described above. Phase-contrast and fluorescence microscopy were performed with a Zeiss Axiophot microscope.

Gel electrophoresis and Western blotting. Cells were washed with PBS and lysed with buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM Pefabloc SC hydrochloride, 5 μg of aprotinin per ml, 20 μM leupeptin, and 1 mM sodium vanadate (radioimmunoprecipitation assay [RIPA] buffer). Insoluble material was pelleted at 4°C for 10 min at 15,000 × *g*. The supernatants were immunoprecipitated with rabbit anti-Raf (sp63) and protein A-Sepharose at 4°C for 2 h, and then the immune complexes were washed several times with RIPA buffer. Proteins were resolved on SDS–7.5% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Blocking of the membranes and subsequent development of the Western blots (immunoblots) with Raf antibodies were performed, as described elsewhere, using chemiluminescence detection methods (Amersham) (29).

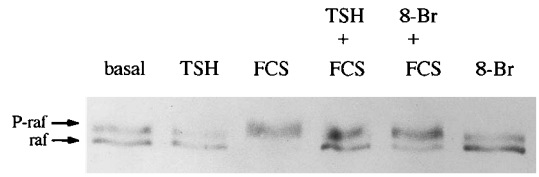

FIG. 1. Western blot analysis of changes in Raf-1 phosphorylation in treated WRT cells. Cells were treated for 0.5 h with the indicated mitogens as described in Materials and Methods. In the case of treatment with more than one mitogen, cells were treated for 30 min prior to treatment with serum. Raf-1 was immunoprecipitated from growth factor-treated cells and subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis as described in Materials and Methods. Lane basal, quiescent WRT cells; lane FCS, 20% fetal calf serum. 8-Br, cAMP analog 8Br-cAMP. These results are representative of three separate experiments which yielded similar results.

RESULTS

TSH stimulation of WRT cells does not result in the hyperphosphorylation of Raf-1. Upon growth factor stimulation of many cell types, Raf-1 becomes markedly hyperphosphorylated, resulting in reduced electrophoretic mobility of the protein on SDS gels (23). MAP kinase was suggested to be responsible for this Raf-1 hyperphosphorylation (59). We examined the phosphorylation state of Raf-1 in TSH-treated cells. Stimulation of cells with TSH for 30 min did not alter the hyperphosphorylation state of Raf-1, as judged by changes in mobility in polyacrylamide gels, compared with that in quiescent WRT cells (Fig. 1, compare lanes basal and TSH). Further, we did not observe changes in Raf-1 gel mobility from as early as 5 min up to 3 h following TSH treatment (data not shown). We also examined the presence of A-Raf and B-Raf but could not detect either of these proteins in WRT cells by Western blot analysis (data not shown). By contrast, serum treatment of WRT cells, under similar conditions, stimulated an increase in Raf-1 phosphorylation (Fig. 1, lane FCS), indicating that these cells are not defective in their abilities to activate the kinase which subsequently phosphorylates Raf-1. These data indicate that mitogenic stimulation of WRT cells with TSH occurs without the stimulation of Raf-1 hyperphosphorylation typically seen upon activation of the Raf-dependent cytoplasmic kinase cascade. In PC12 pheochromocytoma cells, increased intracellular cAMP levels results in the phosphorylation and activation of MAP kinase and yet results in inhibition of activation of both Raf-1 and B-Raf by a variety of growth factors (16, 57, 61). To determine if this was the case in WRT cells, we compared the cellular localization, phosphorylation state, and activity of MAP kinase in cells treated with either serum or TSH. We found that treatment of WRT cells with serum but not TSH stimulated nuclear translocation of, phosphorylation of, and kinase activity towards myelin basic protein by MAP kinase (2, 30). These results suggest that at least two components of the Raf-dependent cytoplasmic kinase cascade, Raf and MAP kinase, are not activated by TSH.

Stimulation of cells with TSH and cAMP analogs reduces Raf-1 hyperphosphorylation and activation of AP-1-regulated genes. Since agents which increase cAMP levels in some cell types inhibit Ras-dependent activation of the Raf-dependent

TABLE 1. Inhibition of wt H-Ras-stimulated gene expression by various treatments

Injected material	Coinjected material or treatment[a]	% β-Gal expression in injected cells[b]	No. of injected cells
wt H-Ras	Rabbit IgG	52 ± 4	744
	K97M-MEK	17 ± 4	331
	Raf antibody	26 ± 6	222
	TSH	7 ± 3	356
	8Br-cAMP	5 ± 3	270
Rabbit IgG	None	3 ± 2	356

[a] Cells were treated with 1 mU of TSH per ml and 0.1 mM 8Br-cAMP.
[b] Error represents the standard error of proportion of data pooled from at least three separate experiments in which controls were always included. β-Gal, β-galactosidase.

TABLE 2. Raf antibodies inhibit wt H-Ras-dependent mitogenesis in Rat1 fibroblasts

Mitogen[a]	Injected material	% BrdU incorporation in injected cells[b]	No. of injected cells
Serum	Mouse IgG	88 ± 2	745
	Raf antibody	43 ± 3	870
Insulin	Mouse IgG	82 ± 2	945
	Raf antibody	31 ± 3	925
EGF	Mouse IgG	45 ± 4	757
	Raf antibody	29 ± 3	868
None	Mouse IgG	23 ± 3	826
	Raf antibody	25 ± 3	884

[a] Cells were treated with growth factors as described in Materials and Methods.
[b] Error represents the standard error of proportion of data pooled from at least three separate experiments in which controls were always included.

cytoplasmic kinase cascade, we examined whether cAMP agonists affected stimulation of Raf-1 hyperphosphorylation by serum. The proportion of Raf found in the hyperphosphorylated state compared with the underphosphorylated state in serum-treated cells was reduced by 80% upon pretreatment of cells with TSH (Fig. 1, lane TSH + FCS) or by simultaneous treatment of cells with TSH and serum (data not shown). Similar results were observed when WRT cells were pretreated with the cAMP analog 8Br-cAMP (75% reduction [Fig. 1, lane 8-Br + FCS]). Treatment of WRT cells with 8Br-cAMP alone, like TSH, had no apparent effect on the phosphorylation state of Raf-1 (Fig. 1, compare lanes basal and 8-Br). We also examined whether cAMP agonists could inhibit activation of MAP kinase by serum. Stimulation of MAP kinase activity by serum was significantly reduced by pretreatment of WRT cells with either TSH or 8Br-cAMP (30). These results rule out the possibility that activation of this MAP kinase occurs independently of Raf-1 activation, as appears to be the case with PC12 pheochromocytoma cells (57).

Activation of Ras typically causes nuclear translocation and activation of MAP kinase, resulting in the phosphorylation and activation of a variety of transcription factors. Such phosphorylation leads to transcription of immediate-early genes, such as those containing AP-1 promoter elements (17). We tested whether TSH had any effect on the expression of genes which contain AP-1 promoter elements by using a WRT cell line which had been stably transfected with a *lacZ* reporter gene under the control of two AP-1 promoter elements (WRT-TRE cells). Injection of purified wt H-Ras protein into WRT-TRE cells resulted in reporter gene expression in 52% of the injected cells (Table 1) compared with 3% in cells injected with only marker IgG. Cells pretreated with TSH prior to injection of wt H-Ras showed significantly reduced expression of the reporter gene (7%; $P < 0.001$). Further, TSH inhibition of wt H-Ras-induced gene expression occurred in a TSH dose-dependent manner (data not shown). 8Br-cAMP also significantly reduced wt H-Ras-induced gene expression to 5% (Table 1), ($P < 0.001$) in a dose-dependent manner (data not shown). These results show that agents which increase cAMP levels in WRT cells inhibit several of the cellular responses typically seen upon activation of the classical cytoplasmic kinase cascade, including hyperphosphorylation of Raf-1, activation of MAP kinase, and expression of AP-1-controlled genes.

To verify that wt H-Ras-induced expression of the reporter gene was dependent on the Raf-dependent cytoplasmic kinase cascade which acts through Raf-1 and MEK, we coinjected purified wt H-Ras into WRT-TRE cells along with either Raf-1 antibody or a catalytically inactive mutant form of MEK-1, a downstream effector of Raf (11, 24, 32). This recombinant protein has a methionine substituted for a lysine at the ATP-binding site of the enzyme, K97M-MEK, rendering it kinase inactive such that it acts as a dominant-negative protein (37). In some cases, MEK appears to be phosphorylated and activated by the Raf-1 kinase, which leads to phosphorylation and activation of the MAP kinases/ERKs by MEK itself. Coinjection of wt H-Ras with Raf-1 antibody significantly reduced wt H-Ras-stimulated gene expression from 52 to 26% (Table 1) ($P < 0.001$), and these data are consistent with the notion that Raf-1 kinase can function downstream of Ras in signal transduction (6). Similarly, coinjection of wt H-Ras with K97M-MEK significantly reduced wt H-Ras-stimulated gene expression to 17% ($P < 0.001$). Coinjection of wt H-Ras with either Raf-1 antibody plus partially purified Raf-1 kinase or K97M-MEK plus purified wt MEK abolished the ability of either Raf-1 antibody or K97M-MEK to reduce wt H-Ras-stimulated gene expression, indicating the specificity of these reagents (data not shown). These data show that in quiescent WRT cells the introduction of wt H-Ras results in expression of genes regulated by AP-1 promoter elements in, at least in part, a Raf-1- and MEK-dependent manner and suggest that in WRT cells, as in some other cell types, agents which increase cAMP levels inhibit signaling through the Raf-dependent cytoplasmic kinase cascade.

TSH-induced DNA synthesis is Raf-1 and MEK independent. TSH signaling in thyroid cells requires both activation of Ras and PKA, and yet activation of PKA inhibits activation of the Raf-dependent cytoplasmic kinase cascade which ultimately is required for Ras-dependent mitogenesis in fibroblasts (44). As previously reported (44), injection of N17 Ras into WRT cells significantly reduced TSH-stimulated DNA synthesis (data not shown). We next investigated whether the Raf-dependent cytoplasmic kinase cascade is required for TSH-induced mitogenesis. We first tested whether an antibody directed against Raf-1 was able to inhibit wt H-Ras-dependent mitogenic signaling when microinjected into fibroblasts. Rat1 fibroblasts were microinjected with the Raf-1 antibody and stimulated with EGF, insulin, or 20% FCS. Microinjection of Raf-1 antibody significantly reduced EGF-, insulin-, and serum-stimulated DNA synthesis (Table 2) ($P < 0.001$). This suggested that Raf-1 antibodies can reduce Raf-dependent signaling by growth factors similarly to inhibition observed following transfection of dominant-negative Raf expression vectors and antisense Raf-1 DNA (23, 28).

We then microinjected the Raf-1 antibody into WRT cells which had been rendered quiescent by hormone starvation for 48 h and examined whether Raf-1 antibody could block cell

TABLE 3. TSH-induced DNA synthesis is Raf and MEK independent

Mitogen[a]	Injected material	% BrdU incorporation in injected cells[b]	No. of injected cells
TSH	Rabbit IgG	61 ± 7	435
	K97M-MEK	59 ± 2	427
	Raf antibodies	69 ± 4	682
wt H-Ras	Rabbit IgG	40 ± 3	678
	K97M-MEK	17 ± 2	1,438
	Raf antibodies	25 ± 2	625
None	Rabbit IgG	10 ± 3	440

[a] Cells were treated with 10 mU of TSH per ml.
[b] Error represents the standard error of proportion of data pooled from at least three separate experiments in which controls were always included.

TABLE 4. TSH pretreatment of cells relieves wt H-Ras of the requirement for Raf and MEK

Injected material	Pretreatment	% BrdU incorporation in injected cells[a]	No. of injected cells[b]
wt H-Ras	None	40 ± 4	678
wt H-Ras + K97M-MEK	None	17 ± 2	1,438
None	None	7 ± 4	NA
wt H-Ras	TSH (0.1 mU)	60 ± 4	554
wt H-Ras + K97M-MEK	TSH (0.1 mU)	65 ± 3	1,199
None	TSH (0.1 mU)	30 ± 4	NA
wt H-Ras	8Br-cAMP (10 μM)	62 ± 4	457
wt H-Ras + K97M-MEK	8Br-cAMP (10 μM)	55 ± 5	405
None	8Br-cAMP (10 μM)	15 ± 5	NA

[a] Error represents the standard error of proportion of data pooled from at least three separate experiments in which controls were always included; control injected cells had similar levels of DNA synthesis compared with uninjected cells.
[b] NA, not applicable.

cycle progression stimulated by TSH. WRT cells microinjected with this antibody showed no significant change in subsequent TSH-stimulated DNA synthesis compared with injected control cells (Table 3). To further explore the role of the Raf-dependent cytoplasmic kinase cascade in TSH-induced signaling, we microinjected WRT cells with K97M-MEK. We found that, consistent with our results with Raf-1 antibody injection, injection of K97M-MEK protein had no effect on TSH-stimulated DNA synthesis (Table 3). These results suggest that TSH-stimulated DNA synthesis is both Raf-1 and MEK independent.

DNA synthesis induced by overexpressed wt H-Ras requires Raf-1 and MEK in WRT cells. One possible explanation for the inability of Raf-1 antibody and K97M-MEK protein to reduce TSH-stimulated DNA synthesis is that in WRT cells Ras signaling may exclusively activate an alternative pathway rather than one which uses Raf-1 and MEK. In order to test this possibility, we coinjected purified wt H-Ras into quiescent WRT cells along with either Raf-1 antibody or K97M-MEK protein and examined the effects of these coinjections on wt H-Ras-induced DNA synthesis. Coinjection of wt H-Ras with Raf-1 antibody significantly reduced wt H-Ras-induced DNA synthesis from 40 to 25% ($P < 0.001$). Coinjection of wt H-Ras with K97M-MEK protein also significantly reduced wt H-Ras-stimulated DNA synthesis from 40 to 17% (Table 3) ($P < 0.001$). These data show that in quiescent WRT cells the introduction of exogenously expressed wt H-Ras results in stimulation of DNA synthesis through a signal transduction pathway which appears to require the activation and use of a Raf-1- and MEK-dependent cytoplasmic kinase cascade. These data also show that signaling through the Raf-dependent cytoplasmic kinase cascade can indeed occur in WRT cells and, therefore, does not appear to be the reason that mitogenic signaling by TSH does not require this kinase cascade.

Pretreatment of WRT cells with TSH relieves the requirement for MEK by overexpressed wt H-Ras. Several lines of evidence suggested to us that Ras might not require use of the Raf-dependent cytoplasmic kinase cascade in TSH-treated WRT cells: (i) exogenously expressed wt H-Ras stimulates expression of an AP-1-controlled reporter gene and DNA synthesis, in quiescent WRT cells, in a Raf-1- and MEK-dependent manner; (ii) TSH treatment of WRT cells inhibits induction of AP-1-controlled gene expression by tetradecanoyl phorbol acetate or wt H-Ras, inhibits induction of c-*jun* expression by serum and insulin-like growth factor 1, reduces stimulation of Raf-1 hyperphosphorylation by serum, and reduces serum stimulation of MAP kinase activity (all hallmarks of activation of the Raf-dependent cytoplasmic kinase cascade [our results and reference 56]); and finally, (iii) mitogenic signaling initiated by treatment of quiescent cells with TSH requires Ras, but not Raf-1 or MEK, both of which are components of the Raf-dependent cytoplasmic kinase cascade. Given these data, we hypothesized that in TSH-stimulated cells, Ras may couple to an effector system other than the Raf-dependent cytoplasmic kinase cascade. To test a part of this hypothesis, we microinjected wt H-Ras protein together with K97M-MEK protein into cells which had been pretreated with a suboptimal dose of TSH and examined the effects on DNA synthesis. If Ras is able to use an alternate signaling system in TSH-treated cells, pretreatment of cells with TSH should relieve the reduction in wt H-Ras-stimulated DNA synthesis observed following coinjection with K97M-MEK. Indeed, we found that DNA synthesis in wt H-Ras and K97M MEK-injected cells significantly increased from 17 to 65% when the cells were treated with TSH 5 min prior to injection (Table 4) ($P < 0.001$). TSH pretreatment of wt H-Ras-injected cells resulted in a significant increase in DNA synthesis from 40 to 60% ($P < 0.001$). DNA synthesis in uninjected cells increased from 7 to 30% in TSH pretreated cells. The effects of TSH on the ability of K97M-MEK to reduce wt H-Ras-stimulated DNA synthesis were dose dependent (data not shown), indicating that this effect requires specific signaling initiated by TSH. We also found that microinjection of the K97M-MEK in the absence of wt H-Ras had no effect on the levels of DNA synthesis seen in TSH-pretreated cells (data not shown). Interestingly, we found that treatment of cells with 8Br-cAMP was able to mimic the effect of TSH and also significantly relieved the reduction in wt H-Ras-stimulated DNA synthesis observed in the presence of K97M-MEK (Table 4) ($P < 0.001$). These data suggest that agents which increase intracellular cAMP levels provide or make available an effector system other than the Raf-dependent cytoplasmic kinase cascade to which Ras may couple, resulting in mitogenic signaling in WRT cells.

DISCUSSION

TSH signaling in thyroid cells requires both Ras- and PKA-dependent signaling pathways. This appears to be a paradox, since in some cell types activation of PKA inhibits coupling of Ras to Raf and hence inhibits activation of the cytoplasmic kinase cascade which includes MEK and MAP kinase (7, 10, 20, 50, 60). Activation of this cytoplasmic kinase cascade is necessary for Ras-dependent signaling in fibroblasts (1). Here

we show that, as was the case for certain other cell types, agents which increase cAMP levels in WRT cells inhibited activation of the well-studied cytoplasmic kinase cascade. TSH pretreatment of cells inhibited serum-stimulated hyperphosphorylation of Raf-1, as well as serum-stimulated MAP kinase activation. Further, pretreatment of cells with TSH inhibited both wt H-Ras- and tetradecanoyl phorbol acetate-induced gene expression from an AP-1-controlled reporter gene (our results and reference 56). Given that MAP kinase activity is inhibited by TSH and MAP kinase activation often results in transcription of AP-1-controlled genes, it seems likely that, as with some other cell types, inhibition of AP-1-controlled gene expression in WRT cells occurs through inhibition of activation of Raf and, subsequently, activation of the Raf-dependent cytoplasmic kinase cascade. Because the inhibitory effects of TSH could be mimicked by the cAMP analog 8Br-cAMP and because inhibition of Raf-1 kinase activity by PKA has recently been well characterized, we suggest that activation of PKA in WRT cells inhibits coupling of Ras to the Raf-dependent cytoplasmic kinase cascade (22).

We also show that despite being Ras dependent, TSH-induced DNA synthesis in WRT cells occurred in a Raf-1- and MEK-independent manner. This was in contrast to DNA synthesis stimulated by wt H-Ras in WRT cells incubated in the absence of TSH, which was Raf-1 and MEK dependent. Hence, wt H-Ras is capable of coupling to and requires the activation and use of Raf-1 and MEK for stimulation of DNA synthesis in WRT cells in the absence of elevations in intracellular cAMP levels. These results are consistent with those of Lamy et al., which show that EGF, but not TSH, stimulates the phosphorylation and nuclear translocation of MAP kinase in primary dog thyrocytes (33). Taken together, these results show that while WRT cells are capable of signaling through the Raf-dependent cytoplasmic kinase cascade, they do not use this signaling cascade subsequent to Ras-dependent mitogenic stimulation with TSH. This suggests the existence of a positive mitogenic signal which requires Ras but not the Raf-1- and MEK-dependent cytoplasmic kinase cascade. This idea is not unprecedented. In *Xenopus* embryos, mesoderm induction by both fibroblast growth factor and activin is Ras dependent, and yet only fibroblast growth factor appears to activate MAP kinase (20, 36). In addition, the *ret* proto-oncogene appears to activate a signaling pathway which requires Ras but which may be Raf and MAP kinase independent (48). Interestingly, *ret* cooperates with Ras in transformation of thyroid cells, and mutations in the *ret* proto-oncogene have been found exclusively in thyroid cells (22, 36).

TSH signaling is Ras dependent but Raf-1 and MEK independent, suggesting to us that Ras signaling in TSH-treated cells might occur through an effector other than Raf. To test that hypothesis, we treated WRT cells with TSH prior to coinjecting wt H-Ras and K97M-MEK protein into the cells. TSH treatment of cells was able to relieve the reduction in wt H-Ras-stimulated DNA synthesis observed in the presence of K97M-MEK. This suggests that Ras might be able to use an effector other than the kinase cascade which occurs through Raf-1 and MEK in TSH-treated cells. While the potential alternate effector system used by Ras in the TSH-pretreated cells remains elusive, preliminary results indicate that pretreatment of cells with TSH followed by microinjection of wt H-Ras and the K97M-MEK under conditions identical to those used above does not result in the induction of genes controlled by either AP-1 promoter elements or cAMP-responsive elements, suggesting that the potential alternate pathway used or activated by Ras does not involve the classical well-studied Ras or cAMP signaling pathways.

While we do not yet know what signaling pathway may be used by Ras in WRT cells, several possible candidates exist. The GTPase-activating proteins, GAP and NF1, both bind to the effector domain of Ras and are postulated to be Ras effectors (46). In preliminary experiments, we have found that microinjection of purified type I GAP protein inhibits DNA synthesis stimulated by both wt H-Ras and TSH, suggesting that this GTPase-activating protein is not functioning as a Ras effector in TSH-stimulated WRT cells. Another potential candidate for downstream signaling by Ras in TSH mitogenic signaling is phosphatidylinositol 3-kinase (PI3K). This kinase phosphorylates phosphatidylinositol at the D-3 position of the inositol ring in response to growth factor stimulation of cells, generating products which are presumed to be involved in signaling (14). PI3K has also been reported to associate with and be activated by Ras, leading to the suggestion that PI3K might be an effector of Ras in some cell types (27, 47, 51). In preliminary experiments we have found that the widely used PI3K inhibitor wortmannin was able to inhibit TSH-stimulated DNA synthesis. Further experiments should clarify the role of PI3K in signaling initiated by both Ras and TSH. RalGDS has also been found to interact specifically with the effector domain of GTP-bound Ras (26). Another potential candidate for downstream signaling by Ras is the phosphatidylcholine-specific phospholipase C (PC-PLC). Overexpression of PC-PLC in NIH 3T3 cells is able to overcome growth inhibition by dominant-negative Ras proteins (9). PC-PLC overexpression appears to activate Raf, however, and therefore is unlikely to play a role in TSH-induced mitogenesis. An isozyme of protein kinase C, PKCζ, was found to play a role in Ras-stimulated *Xenopus laevis* oocyte maturation (13). Furthermore, in fibroblasts this kinase seems to be required for mitogenic signaling and has been proposed to play a role downstream of Ras (5). This isozyme of protein kinase C is insensitive to phorbol esters, Ca^{2+}, and diacylglycerol (43). Phorbol esters are relatively ineffective in the stimulation of DNA synthesis in WRT cells, and calcium fluxes could not be detected in the cells upon stimulation with TSH (40). Thus, PKCζ could be involved in TSH signaling. Additionally, in fibroblasts, Ras activates the recently identified c-Jun NH_2-terminal kinase, a member of the MAP kinase family whose mechanism of activation appears to be distinct from the signaling pathway that leads to MAP kinase activation (12, 42). While stimulation of WRT cell with TSH does not appear to activate c-Jun, c-Jun NH_2-terminal kinase or another as-yet-unidentified kinase may be activated by Ras and play a role in TSH signaling independently of c-Jun activation. Further studies which should shed light on the signaling mechanisms used by TSH and Ras in mitogenic signaling in WRT cells are in progress.

It is interesting to note in Table 4 that the maximal levels of DNA synthesis were observed in wt H-Ras- and K97M-MEK-injected cells treated with suboptimal levels of TSH. It appears as though synergy may be occurring between signaling in untreated wt H-Ras- and K97M-MEK-injected cells and in cells treated with suboptimal levels of TSH. Cooperation between Ras-dependent and cAMP-dependent signaling systems is not unprecedented. In *Saccharomyces cerevisiae*, cAMP levels are regulated by the yeast Ras homologs (55). In fibroblasts, overexpression of c-*src* or stimulation of cells with EGF potentiates the response of cells to cAMP agonists and enhances the production of cAMP in these cells (4, 8). The nature of the synergism between Ras signaling and cAMP signaling in WRT cells should be elucidated by further experiments.

ACKNOWLEDGMENTS

We thank A. Aronheim and A. Lin for helpful advice and discussions and S. J. Mansour, J. Candia, and J. Dela Cruz for expert technical assistance.

This research was supported in part by grants from the NIH (J.R.F., N. Ahn, and J.M.) and the Searle Scholars Program (N. Ahn). N. Al-Alawi was supported by a training grant from NIH to the Biomedical Sciences Graduate Program, UCSD.

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